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**Remarks**

A signed copy of the restriction election accompanies this Response as requested on page 2 of the Office Action.

The coding region corresponds to nucleotides 1–1737 of SEQ ID NO:1, which encode the amino acid sequence of SEQ ID NO:2.

New drawings containing the corrected figure designations, e.g., “Figure 2A”, are submitted herewith.

Claims 25-37 were rejected under 35 U.S.C. 101 on the ground that the claimed invention is not supported by either a credible asserted utility or a well established utility.

Attention is kindly invited to Gross *et al. Biochem. Biophys. Res. comm.* (1994) 205: 1010-1017 (copy enclosed for Examiner's convenience). The publication describes the identification of functional domains in AMP deaminase I from human and rat muscle. Point mutations in the conserved mid and carboxy terminal regions of human AMP deaminase I, revealed sites that are important for catalytic activity or stability of the enzyme and sites that might undergo amino acid substitutions without a detectable effect on enzyme activity.

Furthermore several very well conserved sequence motifs have been identified. For clarification the mutations and motifs mentioned by Gross et al are indicated in Appendix A, which shows a comparison of the amino acid sequence of the claimed sequence from corn (SEQ ID NO:2), a yeast AMP deaminase and AMP deaminase I from human skeletal muscle. The enzymes from yeast and human share 38 and 48 sequence identity, respectively.

The sequence comparison set forth in Appendix A demonstrates that the sequences of the invention share the highly conserved sequence motifs described in Gross et al. (Three of the mutations, indicated by a dot and the corresponding amino acid change, (Phe 266-Leu, Asn 311, His 518-Tyr) did not show a detectable effect on the AMP deaminase activity. Only one of these three mutations, Asn311-Ser, is located in a highly conserved sub-region and represents a conservative amino acid substitution, hence no effect on enzyme activity would be expected. The other four point mutations resulted in an alteration in AMP deaminase activity.

Mutation Asp650-Gly, causes loss of enzymatic activity, although the peptide can still be detected. The latter mutation lies within a highly conserved sequence motif SLSTDDP, which presumably forms part of the active site of AMP deaminase. Mutant Ser516-Pro, which also is located in a highly conserved sub-region of AMP Deaminase I, also yield a peptide with no detectable enzyme activity, however, this mutant does not produce a peptide that is immunologically detectable.

Mutant Glu633-Lys and Gly 577-Glu, both located in highly conserved sub-regions (EPLMEEY and RHPCGEAG, respectively) appear to affect the stability of the enzyme. The sequence motif EPLMEEY has been identified as a Nucleotide binding site based on studies in yeast.

Gross et al. show that comparison of the various members of the AMPD multigene family suggests that the mid and carboxy terminal regions, which are common to all isoforms, contain the catalytic site as well as regulatory sites which are shared among the various isoforms. As can be seen from Appendix A, the mid and carboxy terminal region of the corn, yeast and human AMP deaminases are highly conserved, whereas the N-terminal regions show a high degree of variability which supports the findings in the literature. Accordingly, it is believed that the absence of a start Methionine is not important with respect to completeness and functionality of the disclosed sequence (SEQ ID NO:2).

Furthermore, deletion studies on the yeast enzyme, have shown that deletion of 192 amino acids from the N-terminal region of the protein, does not effect enzymatic activity (Merkler et al. [(1989), J. Biol. Chem. 264, 21422-21430, copy enclosed). Although N-terminal deletions of the rat protein yielded a reduction in enzyme activity (Gross et al.), this appears to be mainly due to the fact, that the N-terminal region from rat skeletal muscle play a crucial role in binding to molecules of Myosin. N-terminal deletions in the rat enzyme seem to substantially affect Myosin binding and, hence, this leads to a reduction of AMP deaminase activity. In view of the above discussion, it is believed that a credible asserted utility for SEQ ID NO:1, encoding SEQ ID NO:2 has been provided.

Accordingly, withdrawal of the rejection of the claims under 35U.S.C.101 is respectfully requested.

Claims 25-37 were also rejected under 35 U.S.C. 112, first paragraph, on the ground that one skilled in the art would not know how to use the claimed invention since a credible or well-established utility appears to be lacking.

It is respectfully submitted that the above comments and amendments are believed to be equally apposite in addressing this ground of rejection. Accordingly, withdrawal of the enablement rejection of the claims under 35 USC §112 is respectfully requested.

Claims 25-37 were rejected under 35 USC §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

It is respectfully submitted that the specification discloses to one of ordinary skill in the art a representative number of AMP deaminases with at least 85%

sequence identity to SEQ ID NO:2, and not just a single polynucleotide encoding SEQ ID NO:2.

The specification, at page 6, line 3 to 16, discloses alterations in nucleotide sequence that are not expected to alter functionality, such as alterations that produce a chemically equivalent amino acid at a given site or alterations in the N- or C-terminal portions. Thus, from the foregoing, the skilled artisan would immediately understand the specification to disclose a representative number of polynucleotide sequences, having different nucleotide substitutions, that encode AMP deaminase but that vary (within 85% sequence identity) of SEQ ID NO:2.

Accordingly, withdrawal of the written description rejection of the claims under 35 USC §112 is respectfully requested.

It is respectfully submitted that the claims are now in form for allowance which allowance is respectfully requested.

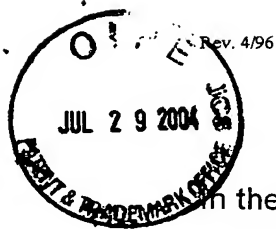
Please charge any fees or credit any overpayment of fees which are required in connection with the filing of this Response, Petition for Extension of Time, Appendix A and Supplemental IDS to Deposit Account No.: 04-1928 (E. I. du Pont de Nemours and Company).

Respectfully submitted,



LYNNE M. CHRISTENBURY  
ATTORNEY FOR APPLICANTS  
REGISTRATION NO.: 30,971  
TELEPHONE NO.: 302-992-5481  
FACSIMILE: 302-892-1026

Dated: July 27, 2004



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:

CASPAR, TIMOTHY ET. AL.

CASE NO.: BB1386 US PCT

APPLICATION NO.: 10/019,633

GROUP ART UNIT: 1638

FILED: DECEMBER 27, 2001

EXAMINER: PHUONG T. BUI

FOR: PURINE METABOLISM GENES IN PLANTS

Commissioner for Patents and Trademarks  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Letter

This is submitted pursuant to a telephonic discussion between the Examiner and Applicant's attorney on Monday, January 5, 2004 regarding the above-identified application.

Remarks

A telephonic discussion between the Examiner and Applicant's Attorney was conducted on Monday, January 5, 2004 regarding the Amendment and Response to Restriction Requirement that was submitted by Certificate of Mailing dated November 3, 2003. The Examiner indicated that this Amendment and Response to Restriction Requirement was unsigned. The Examiner also indicated that since it appeared to be a bona fide attempt to respond to the Office Action dated October 1, 2003 that it would be examined provided that a signed copy of the Amendment and Response to Restriction Requirement was submitted.

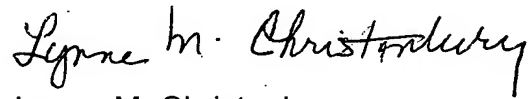
Submitted herewith by facsimile transmission is a signed copy of the aforementioned Amendment and Response to Restriction Requirement that was previously filed by Certificated of Mailing on November 3, 2003.

Applicants apologize for any inconvenience caused by this inadvertent oversight.

Please charge any fees including, but not limited to any extensions of time, associated with the filing of the Amendment and Response to Restriction

Requirement or credit any overpayment to Deposit Account 04-1928 (E. I. du Pont de Nemours and Company).

Respectfully submitted,



Lynne M. Christenbury  
Attorney For Applicants  
Registration No. 30,971  
Telephone: 302-992-5481  
Facsimile: 302-892-1026

Dated: January 5, 2004



PATENT

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In the Application of:

CASPAR, TIMOTHY ET. AL.

CASE NO.: BB1386 US PCT

APPLICATION NO.: 10/019,633

GROUP ART UNIT: 1638

FILED: DECEMBER 27, 2001

EXAMINER: PHUONG T. BUI

FOR: PURINE METABOLISM GENES IN PLANTS

**AMENDMENT AND RESPONSE TO RESTRICTION REQUIREMENT**

Commissioner for Patents and Trademarks

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

This is in response to the Office Action of October 1, 2003, and before examination on the merits, please consider the following remarks:

**Amendments to the Specification** begin on page 2 of this paper.

**Amendments to the Claims** are reflected in the listing of the claims which begins on page 3 of this paper.

**Remarks** begin on page 5 of this paper.

**AMENDMENTS TO THE SPECIFICATION:**

Please amend the specification as follows:

**Paragraph bridging pages 8-9, lines 36-37 and 1-10, respectively:**

Amino acid and nucleotide sequences can also be evaluated either manually by one skilled in the art, or by using computer-based sequence comparison and identification tools that employ algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.* 215:403-410[~~;- see also~~ [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)]). In general, a sequence of ten or more contiguous amino acids or thirty or more contiguous nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12 or more nucleotides may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers.

**Paragraph at page 21, lines 25-41:**

cDNA clones encoding AMP or adenosine deaminases were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.* 215:403-410[~~;- see also~~ [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)]) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States (1993) *Nat. Genet.* 3:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.



**AMENDMENTS TO THE CLAIMS:**

This listing of claims will replace all prior versions, and listings, of claims in the application:

**Listing of Claims:**

Claims 1-24 (canceled)

25 (new): An isolated polynucleotide comprising:

- (a) a nucleotide sequence encoding a polypeptide having AMP deaminase activity, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO:2 have at least 85% sequence identity based on the Clustal V alignment method, or
- (b) the complement of the nucleotide sequence, wherein the complement and the nucleotide sequence contain the same number of nucleotides and are 100% complementary.

26 (new): The polynucleotide of Claim 25, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO:2 have at least 90% sequence identity based on the Clustal alignment method.

27 (new): The polynucleotide of Claim 25, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO:2 have at least 95% sequence identity based on the Clustal alignment method.

28 (new): The polynucleotide of Claim 25, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:2.

29 (new): The polynucleotide of Claim 1, wherein the nucleotide sequence comprises the nucleotide sequence of SEQ ID NO:1.

30 (new): A vector comprising the polynucleotide of Claim 25.

31 (new): A virus comprising the isolated polynucleotide of Claim 25.

32 (new): A recombinant DNA construct comprising the polynucleotide of Claim 1 operably linked to at least one regulatory sequence.

33 (new): A method for transforming a cell comprising transforming a cell with the polynucleotide of Claim 25.

34 (new): A cell comprising the recombinant DNA construct of Claim 32.

35 (new): A method for producing a plant comprising transforming a plant cell with the polynucleotide of Claim 25 and regenerating a plant from the transformed plant cell.

- 36 (new): A plant comprising the recombinant DNA construct of Claim 32.
- 37 (new): A seed comprising the recombinant DNA construct of Claim 32.

**REMARKS**

The specification has been amended in two places on pages 8-9 and page 21, to remove reference to the following URL: [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/).

Claims 25-37 are pending. Claims 1-24 have been canceled. New Claims 25-37 are meant to replace original Claims 1-9, 11-13, 16, and 18-22, drawn to a polynucleotide and first method of using. The term "recombinant DNA" is used to replace "chimeric gene" from the original claims, support for which can be found on page 12, lines 28-31. Support for the percent identities cited in claims 25-27 can be found on page 7, lines 18-24. Thus, no new matter has been added.

**RESPONSE TO RESTRICTION REQUIREMENT**

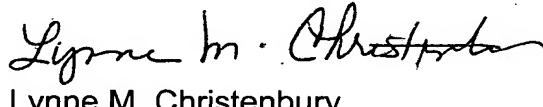
Applicants hereby provisionally elect the subject matter of Group I, claims 1-9, 11-13, 16, and 18-22, and as required under 37 CFR 1.499, a further election of invention (A) is made to a polynucleotide of SEQ ID NO:1 or a corresponding polypeptide of SEQ ID NO:2, without traverse, for further prosecution in the instant application subject to Applicants' right to pursue the non-elected subject matter in a divisional application or applications pursuant to 35 USC §121.

It is believed that new claims 25-37 correspond to claims 1-9, 11-13, 16, and 18-22 which constituted the subject matter of Group I.

In view of the foregoing amendments and remarks, it is respectfully submitted that this case is in form for allowance which allowance is respectfully requested.

Please credit any overpayment or charge any fees associated with the filing of this response or credit any overpayment to Deposit Account 04-1928 (E. I. du Pont de Nemours and Company).

Respectfully submitted,



Lynne M. Christenbury

Attorney For Applicants

Registration No. 30,971

Telephone: 302-992-5481

Facsimile: 302-892-1026

Dated: January 5, 2004

## Appendix A

Appendix A shows a comparison of the amino acid sequences of AMP deaminase from corn clone with SID p0091.cqrav79r (SEQ ID NO:2), yeast and human set for the in NCBI General Identifier No's: 1077011 and 4557311, respectively. Amino acids conserved among all sequences are indicated with an asterisk (\*) on the top row; dashes are used by the program to maximize alignment of the sequences. Mutations described in Gross et al. [(1994), Biochem. Biophys. Res. Comm. 205: 1010-1017], are designated by a dot (•) and the corresponding amino acid change. Motifs related important for activity or stability as described in Gross et al. are underlined.

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                                *
SEQ ID NO:2      -----PRVR-----VAPWEKEVIND
Gi:1077011      MDNQATQRLNDLSLEPAPSHDEQDGSGLVIDIDQRKIGDEQAGVVDDDETPPLEQQDSHE
Gi:4557311      M-----PLFKLPAAEEKQIDDAMR-NFAEKVFASEVKDEGGRQEISPFVDVEIC-

SEQ ID NO:2      PCTPKPNPNPFTYVPEPK-----
Gi:1077011      SLAADSRNANFSYHENQQLENGTKQLALDEHDSHSAILEQPSHSTNCSSSNIAAMNKGH
Gi:4557311      -----PISHHEMQAHIFHLETLS-TEARRKKRFQGR

SEQ ID NO:2      --SEHVFQTVDG-----
Gi:1077011      DSADHASQNSGGKPRTLSSASAQHILPETLKSFAGAPVVNKQVRTSASYKMGLADDASQO
Gi:4557311      KTVNLSIPLSETSSTKLSHIDEYISSP--TYQTVPDFQR-VQITGDYASGVTVED----

                                *      *
SEQ ID NO:2      -----VI--HVIAD-KDCTE-----SIYP-----
Gi:1077011      FLDDPSSSELI--DLYSKVAECRNLRACYQTISVQNDQNP-----NKPQWVVPVPPPK
Gi:4557311      F-----EIVCKGLYRAL--C--IREKYMOKSFQRFPKTPSKYLRNIDGEAWV-----AN

SEQ ID NO:2      -----
Gi:1077011      PSYNSDTKTVPVPTNKPDAEVDFDKCEIPGEDPDWEFTLNDDDSYVVRSG---KTDEL
Gi:4557311      ESFYP----VFTPPVKKGEDPF---RTDNLPENLGYHLKMKDGVVYVYPNEAAVSKDEPK

                                *      *      **      *      **      *      *      *
SEQ ID NO:2      ---VADATTFFTDLHYILRVTAAGNTRTVCHNRLNLEHKFKFHLMLNADREFLAQKTAP
Gi:1077011      IAQIPTLRDYLLDLEKMISISSDGPAKSFAYRRLQYLEARNLYLLNEYQETSVSQRNP
Gi:4557311      PLPYPNLDTFLDDMNFLALIAQGPVKTYTHRRLLKFLSSKFQVHQLNEMDELKELKNNP

                                •L
*****  *****  *  *****  *****  *  *  *****  *  *  *
SEQ ID NO:2      HRDFYNVRKVDTHVHHSACMNQKHLLRFIKSKLRKEPDEVVIFRDGTYMTLKEVFESLDL
Gi:1077011      HRDFYNVRKVDTHVHHSACMNQKHLLRFIKHKLRSKDEKVIIFRDGKLLTLDEVFRSLHL
Gi:4557311      HRDFYNCRKVDTHIHAAACMNQKHLLRFIKKSYQIDADRVVYSTKEKNLTLELFAKLKM

                                •S
***  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
SEQ ID NO:2      TGYDLNVDLLDVHADKSTFHRFDKFNLYNPGQSRLEIFLKQDNLIQGRFLAELTKQV
Gi:1077011      TGYDLSIDTLDMAHAKDTFHRFDKFNLYNPIGESRLREIFLKTNNYIKGTYLADITKQV
Gi:4557311      HPYDLTVDSLVDHAGRQTFQRFDKFNDKYNPVGASELRDLYLKTNDYINGEYFATIIKEV

**      ***  *  *  *  *  *  *  *  *  *  *  *  *  *
SEQ ID NO:2      FSDLSASKYQMAEYRISYGRKQSEWDQLASWIVNNELHSGNVVWLQVIPRLYNVYKEMG
Gi:1077011      IFDLENSKYQNCYRISVYGRSLDEWDKLASWVIDNKVISHNVRWLQVIPRLYDIYKKTG
Gi:4557311      GADLVEAKYQHAEPRLSIYGRSPDEWSKLSSWFVCNRIHCPNMTWMIQVPRIYDVFRSKN

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# Appendix A (Continued)

SEQ ID NO:2	IVTSFQNLLDNIFVPLFEVTIDPASHPQLHVFLKQVVGLDLVDDESK-PERRPTKHMPTP
Gi:1077011	IVQSFQDICKNLFQPLFEVTKNPQSHPKLHVFLQRVIGFDSVDDESK-VDRRFHRKYPKP
Gi:4557311	FLPHFGKMLENIFMPVFEATINPQADPELSVFLKHITGFD <u>SVDDDESK</u> HSGHMFSSKSPKP
	●P ●Y
SEQ ID NO:2	EQWTNVFNPAFSYYAYCYANLFTLNKLRESKGMTTIKFRPHAGEAGDVDHLAATFLLCH
Gi:1077011	SLWEAPQNPPYSYYLYLYSNVASLNQWRAKRGFNTLVLRPHCGEAGDPEHLVSAYLLAH
gi4557311	QEWTLKKNPSYTYAYMYANIMVLNSLRKERGMNTFLFR <u>PHCGEAGAL</u> THLMTAFMIAD
	●E
SEQ ID NO:2	NISHGINLRKSPVLQYLYYLQGIGLAMSPLSNNSLFLDYHRNPFPFTFFQRLNVSLSTDD
Gi:1077011	GISHGILLRKVPFVQYLYYLDQVGIAMSPLSNNALFLTYDKNPFPRYFKRGLNVSLSTDD
Gi:4557311	DISHGLNLKKSPVLQYLFFLAQIPIAMSPLSNNSLFL <u>EYAKNPFLD</u> FLQKGLMISLSTDD
	●G
SEQ ID NO:2	PLQIHLTKEPLVEEYSIAASLWKLSSCDLCEIARNSVYQSGFSHALKAHWIGKNYFKRGP
Gi:1077011	PLQFSYTREPLIEEYSVAAQIYKLSNVDMCELARNSVLQSGWEAQIKKHGIGKDFDKSGV
Gi:4557311	<u>PMQFHFTKEPLMEEYAIAA</u> QVFKLSTCDMCEVARNSVLQCGISHEEKVKFLGDNYLEEGP
	●K
SEQ ID NO:2	AGNDIHRTNVPHIRVQFREMIWRNEMKLV--YSDNEILIPDELDL
Gi:1077011	EGNDVVRTNVDPDIRINYRYDTLSTELELVNHFANFKRTIEEK---
Gi:4557311	AGNDIRRTNVAQIRMAYRYETWCYELNLIAE-----GLKSTE

## AMP Deaminase from Yeast

### ROLE IN AMP DEGRADATION, LARGE SCALE PURIFICATION, AND PROPERTIES OF THE NATIVE AND PROTEOLYZED ENZYME\*

(Received for publication, May 15, 1989)

David J. Merkler†, Anupam S. Wali, James Taylor, and Vern L. Schramm§

From the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461 and the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Eukaryotes have been proposed to depend on AMP deaminase as a primary step in the regulation of intracellular adenine nucleotide pools. This report describes 1) the role of AMP deaminase in adenylate metabolism in yeast cell extracts, 2) a method for large scale purification of the enzyme, 3) the kinetic properties of native and proteolyzed enzymes, 4) the kinetic reaction mechanism, and 5) regulatory interactions with ATP, GTP, MgATP, ADP, and  $PO_4$ . Allosteric regulation of yeast AMP deaminase is of physiological significance, since expression of the gene is constitutive (Meyer, S. L., Kvalnes-Krick, K. L., and Schramm, V. L. (1989) *Biochemistry* 28, 8734-8743).

The metabolism of ATP in cell-free extracts of yeast demonstrates that AMP deaminase is the sole pathway of AMP catabolism in these extracts. Purification of the enzyme from bakers' yeast yields a proteolytically cleaved enzyme,  $M_r$  86,000, which is missing 192 amino acids from the N-terminal region. Extracts of *Escherichia coli* containing a plasmid with the gene for yeast AMP deaminase contained only the unproteolyzed enzyme,  $M_r$  100,000. The unproteolyzed enzyme is highly unstable during purification.

Substrate saturation plots for proteolyzed AMP deaminase are sigmoidal. In the presence of ATP, the allosteric activator, the enzyme exhibits normal saturation kinetics. ATP activates the proteolyzed AMP deaminase by increasing the affinity for AMP from 1.3 to 0.2 mM without affecting  $V_M$ . Activation by ATP is more efficient than MgATP, with half-maximum activation constants of 6 and 80  $\mu$ M, respectively. The kinetic properties of the proteolyzed and unproteolyzed AMP deaminase are similar. Thus, the N-terminal region is not required for catalysis or allosteric activation.

AMP deaminase is competitively inhibited by GTP and  $PO_4$  with respect to AMP. The inhibition constants for these inhibitors decrease in the presence of ATP. ATP, therefore, tightens the binding of GTP,  $PO_4$ , and

AMP. The products of the reaction,  $NH_3$  and IMP, are competitive inhibitors against substrate, consistent with a rapid equilibrium random kinetic mechanism. Kinetic dissociation constants are reported for the binary and ternary substrate and product complexes and the allosteric modulators.

AMP deaminase (EC 3.5.4.6) catalyzes the hydrolytic cleavage of AMP to IMP and  $NH_3$ . It is found in a variety of eukaryotes (1-5), but no prokaryote has been demonstrated to contain a specific AMP deaminase (6). The enzyme has been proposed to play a role in the maintenance of adenylate energy charge (7-9) and is one of the three enzymes which comprise the purine nucleotide cycle (10). The operation of the purine nucleotide cycle is important for the interconversion of adenine and guanine nucleotides (11), for the production of ammonia, for energy production by increasing the levels of tricarboxylic acid cycle intermediates during exercise (12), and for regulating intracellular levels of AMP. Humans deficient in muscle AMP deaminase have a decreased capacity for work, exhibit post-exertional nucleotide imbalances, and do not release ammonia from muscle during exercise (13, 14).

Regulation of the adenine nucleotide pool by AMP degradation has been demonstrated to occur both in prokaryotes (15-17) and in eukaryotes (6, 7). This regulation is proposed to occur by AMP nucleosidase ( $AMP + H_2O \rightarrow$  adenine + ribose 5-phosphate) in prokaryotes (17), whereas in eukaryotes AMP deaminase is the regulatory enzyme (7, 18). In contrast to AMP deaminase, AMP nucleosidase has been detected in a number of prokaryotes but not in eukaryotes (17, 18). Both of these enzymes are allosterically activated by ATP and inhibited by inorganic phosphate, suggesting similar metabolic roles. However, analysis of the DNA and deduced amino acid sequences of yeast AMP deaminase and *E. coli* AMP nucleosidase have indicated that they have evolved from distinct precursors (19).

The role of AMP deaminase in yeast metabolism has not yet been firmly established. Recent studies with a mutant yeast deficient in the enzyme grew at near normal rates. Regulation of the enzyme must occur by substrate and allosteric controls, since the 5'-regulatory region indicates constitutive expression of a single gene in yeast. These studies were confirmed by growth of yeast under a variety of nutritional conditions (19). To investigate the regulation of AMP deaminase activity in yeast, we have studied the pathway of AMP degradation in cell free yeast preparations. The enzyme has been purified to homogeneity on a large scale. Its kinetic properties, kinetic reaction mechanism, and allosteric regulation have been defined.

The enzyme prepared from yeast has undergone the prote-

\* This work was supported by National Institutes of Health Research Grants GM21043 and GM36604 (to V. L. S.) and National Institutes of Health Postdoctoral Fellowship GM10599 (to D. J. M.). Preliminary reports of this work have been presented at the 1980 Annual Meeting of the American Society of Microbiology, Abstr. K124 and the 1986 American Society of Biologic Chemistry Meeting (*Fed. Proc.* 45, Abstr. 133). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Unigene Laboratories, Inc., Division of Protein Chemistry, 110 Little Falls Rd., Fairfield, NJ 07006.

§ To whom correspondence should be addressed: Dept. of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

olytic loss of 192 amino acids from the N terminus. The proteolyzed enzyme is compared to the native enzyme prepared by expression in *E. coli*.

## EXPERIMENTAL PROCEDURES

### Materials

Fresh Red Star yeast were obtained from local suppliers and used immediately. Phosphocellulose (Selectacel, reagent grade) was a product of James River Corp., Berlin, NH. All nucleotides, nucleosides, ribose 5-phosphate, and ribose were products of either P-L Biochemicals or Sigma.  $[8-^{14}\text{C}]\text{ATP}$ ,  $[8-^{14}\text{C}]\text{adenosine}$ , and uniformly labeled  $[^{14}\text{C}]\text{AMP}$  were purchased from Du Pont-New England Nuclear.  $[8-^{14}\text{C}]\text{IMP}$  was from Amersham Corp. Antisera against AMP nucleosidase from *Azotobacter vinelandii* was prepared in rabbits (20). Affinity-purified rabbit antisera against yeast AMP deaminase was a gift of Dr. S. L. Meyer of this laboratory. Goat anti-rabbit IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, sodium nitroprusside, pyruvate kinase, myokinase, benzamidine, leupeptin, pepstatin A, and soybean trypsin inhibitor were from Sigma. Purified AMP nucleosidase from *E. coli* and *A. vinelandii* were gifts of Dr. D. W. Parkin of this laboratory. Lead-free glass beads (0.5–0.7-mm diameter) were from Glen Mills, Inc., Maywood, NJ. All other materials were of the highest quality commercially available. Water was deionized and distilled.

AMP deaminase was expressed in *E. coli* HB101 containing a plasmid encoding the yeast AMP deaminase. The construction and properties of this organism have been reported (19) and has been named *E. coli* HB101 (EBS-6).

### Adenylate Degradation Experiments

Cell extracts were prepared from yeast cake by the addition of 1 ml of buffer containing 20 mM potassium phosphate, pH 7.0, 400 mM KCl, 5% glycerol, 1 mM DTT, and 3  $\mu\text{M}$  phenylmethylsulfonyl fluoride/g cells. The mixture was treated with an Aminco pressure cell at 15,000–20,000 p.s.i. and extracts clarified by centrifugation. Assay mixtures containing radioactive ATP or other nucleotides were initiated by the addition of yeast extract and incubated at 30°C in the presence of the extraction buffer. At the appropriate times, samples (20  $\mu\text{l}$ ) were placed in 1 ml of 67% ethanol, heated for 3 min at 80°C, and placed on ice. Solvent was removed under vacuum and 250  $\mu\text{l}$  of water added. The solution was clarified by centrifugation and ATP, ADP, AMP, IMP, ribose 5-phosphate, inosine, hypoxanthine, adenosine, adenine, and ribose were separated by two-dimensional chromatography (21) and quantitated by scintillation counting.

### AMP Deaminase Assays

Enzyme activity was measured spectrophotometrically at 235 or 260 nm using extinction coefficients of  $2.25 \text{ mM}^{-1} \text{ cm}^{-1}$  or  $\sim 8.40 \text{ mM}^{-1} \text{ cm}^{-1}$ , respectively. The light path was 1 cm or 0.5 mm depending on AMP concentration. Reactions at 30°C were initiated by 1–20  $\mu\text{l}$  of enzyme into reaction mixtures containing 50 mM triethanolamine HCl, pH 7.0, 0.1 M KCl, 0.1 mM DTT, and the appropriate concentrations of AMP and ATP.

Deamination of AMP was also assayed by the colorimetric determination of ammonia (22). Reactions were initiated with 1–15  $\mu\text{l}$  of enzyme into 10 mM HEPES- $\text{K}^+$ , pH 7.0, 0.1 M KCl, 0.1 mM DTT, and the appropriate concentrations of AMP and ATP. HEPES could be replaced by triethanolamine HCl. Initial reaction rates were determined from ammonia formation in two or four time periods. Reactions were terminated by the addition of the phenol/nitroprusside reagent and color was developed at 37°C for 30 min after addition of the alkaline/hypochlorite reagent.

Initial reaction rates at high nucleotide or  $\text{NH}_4\text{Cl}$  concentration were determined by HPLC. Conditions were as described for the colorimetric assay, except that reactions were terminated by the addition of 50  $\mu\text{l}$  of 1.2 M HCl. Before HPLC analysis, solutions were neutralized by the addition of NaOH and an aliquot injected onto a reverse phase  $\text{C}_{18}$   $\mu\text{Bondapak}$  column (0.2  $\times$  30 cm, flow rate 2.0 ml/min), and percent conversion determined by integration of the peaks

at 248.5 nm (the isobestic point for AMP and IMP). The solvent system was 0.1 M ammonium phosphate pH 5.0.

### Large Scale Purification of AMP Deaminase

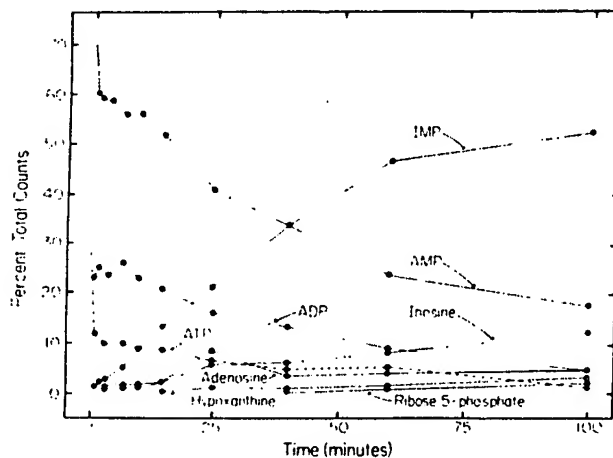
All steps were carried out at 4°C.

**Extraction**—80 pounds of Red Star yeast were suspended in 12 liters of 20 mM potassium phosphate, pH 7.0, 0.3 M KCl, 0.1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 50  $\mu\text{g}/\text{ml}$   $\text{NaN}_3$  (Buffer A). The yeast were disrupted (rate, 5–10 liter/h) in a continuous flow Impandex Dyano-Mill filled with 0.5–0.7-mm glass beads. The resulting solution was diluted with an equal volume of 20 mM potassium phosphate, pH 7.0, 0.1 mM DTT, 50  $\mu\text{g}/\text{ml}$   $\text{NaN}_3$  (Buffer B) and centrifuged to remove cellular debris.

**Batchwise Phosphocellulose Treatment**—Phosphocellulose, equilibrated in Buffer B containing 0.45 M KCl, was added to the supernatant from the previous step (volume supernatant/volume packed phosphocellulose = 8) and stirred for 6–12 h. The phosphocellulose was collected by filtration (Nitex fine mesh nylon sheets, Tetko, Inc., Elmsford, NY) and resuspended in Buffer B containing 0.45 M KCl. The mixture was washed with 6 liters of Buffer B containing 0.45 M KCl in a Büchner funnel (12  $\times$  26 cm). AMP deaminase was eluted by washing the resin with Buffer B containing 1.5 M KCl. Fractions (500 ml) were collected until the eluant was colorless. Active fractions were combined and diluted with Buffer B to give a final KCl concentration of 0.45 M.

**Phosphocellulose Chromatography, KCl**—The solution from the previous step was loaded on a column of Sephadex G-25 mixed with phosphocellulose (8  $\times$  25 cm, 1:1 wet volume) equilibrated with Buffer B containing 0.3 M KCl. AMP deaminase was eluted with a linear gradient of 0.3–1.8 M KCl in Buffer B (total volume = 7 liters). The active fractions were combined.

**Phosphocellulose Chromatography, ATP**—The pooled fractions from the previous step were diluted with Buffer B to give 0.4 M KCl. The resulting solution was loaded onto a column of Sephadex G-25: phosphocellulose column (3.5  $\times$  23 cm, 1:1 wet volume) equilibrated in Buffer B containing 0.3 M KCl. AMP deaminase was eluted with a linear gradient of 0–5 mM ATP in Buffer B containing 0.3 M KCl (total volume = 2 liters). Active fractions were combined. Fractions with a specific activity  $> 500 \mu\text{mol}/\text{min}/\text{mg}$  were concentrated to  $\geq 5 \text{ mg}/\text{ml}$  protein, dialyzed into Buffer B containing 0.3 M KCl, and stored at  $-80^\circ\text{C}$ . Fractions with specific activities between 100 and 500  $\mu\text{mol}/\text{min}/\text{mg}$  could be purified to homogeneity by gel filtration on Ultrolgel AcA 22 or by repeating phosphocellulose chromatography with ATP elution.



**FIG. 1. Degradation of AMP in the presence of ATP.** Yeast extract (20  $\mu\text{l}$ ), prepared as described under "Methods," was added to 480  $\mu\text{l}$  of assay mixture containing 65 mM triethanolamine HCl, pH 7.0, 5 mM AMP, 2 mM ATP, 8 mM  $\text{MgCl}_2$ , 100 mM KCl, 1  $\mu\text{Ci}$  of uniformly labeled  $[^{14}\text{C}]\text{AMP}$ , and 0.4  $\mu\text{Ci}$  of  $[8-^{14}\text{C}]\text{ATP}$ . At the indicated time, aliquots (20  $\mu\text{l}$ ) were removed and analyzed for nucleotide content as described under "Methods." Note that the specific radioactivity of the AMP and ATP were equal during the experiment. Under these conditions the ordinate scale is also proportional to the concentration of nucleotides.

<sup>1</sup> The abbreviations used are: DTT, dithiothreitol; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

Partial Purification of Large M<sub>r</sub> AMP Deaminase

**Extraction**—*E. coli* HB101 (EBS-6), which expresses yeast AMP deaminase (19), was grown overnight at 37°C in Luria broth containing 60 µg/ml ampicillin. The *E. coli* cells (4 g) were suspended in 4 ml of 20 mM potassium phosphate, pH 7.0, 20 mM KCl, 25 mM benzamidine, 0.5 mM DTT and broken in a Braun homogenizer model 2876. After the cells were disrupted, the sample was adjusted to 430 µM phenylmethylsulfonyl fluoride, 14 µM leupeptin, 43 µM pepstatin A, and 0.7 µg/ml soybean trypsin inhibitor and clarified by centrifugation.

**Ammonium Sulfate Precipitation**—Protein precipitating between 20 and 35% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was collected by centrifugation. The pellet was suspended in 0.5 ml of 20 mM triethanolamine HCl, pH 7.0, 20 mM KCl, 5 mM benzamidine, 0.5 mM DTT and stored at -80°C.

## Protein Concentration

Protein was determined by the Bradford method (23) using bovine serum albumin as a standard. The absolute concentration of purified AMP deaminase was determined by dry weight analysis after dialysis into 5 mM potassium phosphate, pH 7.0, 300 µg/ml NaN<sub>3</sub>. Samples of the dialysate and enzyme (approximately 13 mg/ml) were placed on aluminum discs, dried to constant weight, and the weights determined on a Cahn model M-10 microbalance. Duplicate analyses agreed to within 2%. The average of three such determinations was used to establish the protein concentration. The UV spectra of enzyme solutions with known protein concentration showed a maximum absorption at 280 nm with A<sub>1%</sub><sup>1</sup> = 17.0.

## Gel Chromatography and Immunodetection

Purified proteins and cell extracts were separated by SDS-polyacrylamide gel electrophoresis (24) and electrotransferred to nitrocellulose (25). AMP deaminase was immunologically detected using affinity-purified rabbit antiserum raised against proteolyzed AMP deaminase (19) and the antibody reaction visualized by incubating the nitrocellulose first with goat anti-rabbit IgG-alkaline phosphatase conjugate diluted 1:1000 in 67 mM potassium phosphate, pH 6.7, 0.14 M KCl, 0.5% (v/v) Tween 20 followed by incubation with 5-bromo-4-chloro-3-indolyl phosphate as described by Blake *et al.* (26). Protein molecular weight standards in the molecular weight range 20,000–116,000 were purchased from Sigma.

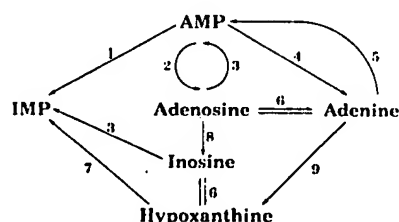
## Data Analysis

Linear double plots were fit to Equation 1 (27).

$$v = \frac{V_M[S]}{K_M + [S]} \quad (1)$$

Kinetic data which resulted in curvilinear double-reciprocal plots were fitted to Equation 2 using an iterative procedure.

$$v = \frac{V_M[S]^n}{K' + [S]^n} \quad (2)$$



SCHEME I. The pathway of AMP degradation. The numbers represent AMP deaminase (1), AMP nucleotidase and/or alkaline phosphatase (2), nucleoside kinase (3), AMP nucleosidase (4), adenine phosphoribosyl transferase (5), purine nucleoside phosphorylase (6), hypoxanthine phosphoribosyl transferase (7), adenosine deaminase (8), and adenine deaminase (9).

Experiments which resulted in competitive inhibition were fitted to Equation 3 (27).

$$v = \frac{V_M[S]}{K_M \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (3)$$

## RESULTS

**AMP Catabolism in Yeast Cell Extracts**—Yeast extracts catalyze the conversion of [<sup>14</sup>C]AMP to [<sup>14</sup>C]IMP in the presence of ATP (Fig. 1). When ATP was omitted or when P<sub>i</sub>, an inhibitor of yeast AMP deaminase (5, 18, 28), was present at high concentrations, the formation of IMP from AMP was decreased by more than 70%. The most direct route of IMP formation from AMP is by the action of AMP deaminase; however, IMP could also be formed by the combined actions of other enzymes as shown in Scheme I. Thus, the actions of AMP nucleosidase, adenine deaminase, and hypoxanthine phosphoribosyl transferase; of AMP nucleotidase, adenosine deaminase, and nucleoside kinase; or of AMP nucleotidase, adenosine deaminase, purine nucleoside phosphorylase, and hypoxanthine phosphoribosyl transferase would result in IMP formation. No activity or cross-reacting material with rabbit antiserum prepared against AMP nucleosidase from *A. vinelandii* could be detected. To test more directly for AMP nucleosidase activity, extracts were tested for the ability to form reducing sugar from AMP in the presence or absence of ATP. No activity was detected. These results indicated the absence of AMP nucleosidase. To test for the presence of the pathway involving adenosine or inosine, experiments similar to that of Fig. 1 used a low concentration of high specific radioactivity [8-<sup>14</sup>C]adenosine and the usual concentrations of unlabeled AMP and ATP. If adenosine or inosine are intermediates for IMP formation, the product IMP would be radioactive. These experiments gave no significant counts in IMP, indicating that neither adenosine or inosine are intermediates in the pathway. Based on these results, AMP deaminase is the major pathway of AMP catabolism in yeast extracts in the presence of ATP.

**Purification of Proteolyzed AMP Deaminase**—The large scale purification of AMP deaminase from 80 pounds of commercial bakers' yeast is summarized in Table IA. The yield of enzyme is approximately 30%. A specific activity of 600–700 µmol/min/mg is higher than the value of 300–400 µmol/min/mg reported previously for homogenous yeast AMP deaminase (5, 28).<sup>2</sup> The purity of the enzyme is ≥95% by SDS-polyacrylamide gel electrophoresis (Fig. 2A). This purification scheme provides enzyme which has a subunit molecular weight of 86,000 equivalent to that described by other investigators (28). As shown below, this form has a lower molecular weight than enzyme present in actively growing yeast (19) or that purified from an expression system in *E. coli* and prepared in the presence of high concentrations of protease inhibitors (Fig. 2B, compare lanes 1–3 with lane 4).

**Purification of Large AMP Deaminase**—The partial purification of yeast AMP deaminase expressed in *E. coli* HB101 (EBS-6) is summarized in Table IB. Attempts to purify the large AMP deaminase from yeast or *E. coli* resulted in a rapid loss of activity. Attempts to stabilize the large AMP deaminase during further purification using a variety of protease inhibitors, rapid purification steps, or excess EDTA have not been successful. Although the purity of the large AMP deaminase is only 2–3% assuming a specific activity of 800, less

<sup>2</sup> Specific activities as high as 886 µmol/min/mg have been obtained. Purified AMP deaminase is most stable at -80°C, but gradually loses specific activity on repeated freeze-thaw cycles.



TABLE I  
Purification of AMP deaminase

Step	Volume	Protein	Units	Specific activity
A. Purification from baker's yeast (proteolytically cleaved form)				
	ml	mg	$\mu\text{mol/min}$	units/mg
Extract	63,300	1,620,000	194,000	0.1
Batch phosphocellulose	6,060	16,500	78,400	4.8
Phosphocellulose-KCl	905	3,500	76,000	21.9
Phosphocellulose-ATP No. 1				
Fraction A <sup>a</sup>	118	48	35,000	719
Fraction B <sup>b</sup>	302	293	29,500	101
Phosphocellulose-ATP No. 2				
Fraction B	285	43	25,700	602
B. Purification of cloned yeast enzyme <i>E. coli</i> (EBS-6) <sup>c</sup> (large form)				
Extract	7.0	150	360	2.4
Ammonium sulfate ppt	0.6	13	245	18.8

<sup>a</sup> Fraction A consisted of the peak fractions from the ATP gradient elution of the phosphocellulose column.

<sup>b</sup> Fraction B consisted of the enzyme fractions with low specific activity from the phosphocellulose column. The ATP was removed by dialysis and the ATP elution step repeated.

<sup>c</sup> *E. coli* were obtained from a 2-liter culture of strain HB101 (EBS-6) which expresses yeast AMP deaminase (19).

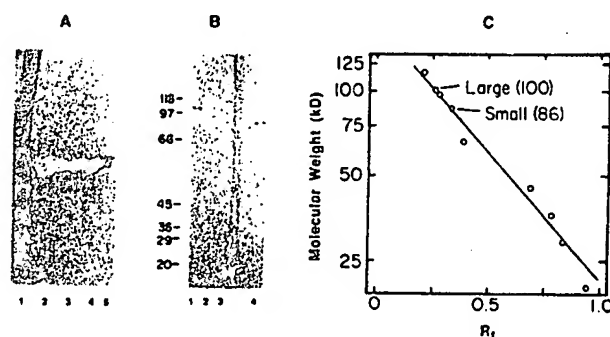


FIG. 2. Western blot of yeast AMP deaminase samples. Purified yeast AMP deaminase was electrophoresed on SDS polyacrylamide gels (7.5%), transferred to nitrocellulose, and stained with Amido-Schwartz (A). Immunoblotting analysis of proteins transferred to nitrocellulose is shown in B. The rabbit anti-AMP deaminase (prepared against the proteolyzed enzyme) was visualized with goat anti-rabbit IgG alkaline phosphatase conjugate (26). A, lane 1, shows yeast extract (37  $\mu\text{g}$ ). Lanes 2-5 contained purified AMP deaminase (proteolyzed) as follows: lane 2 = 29  $\mu\text{g}$ , specific activity 886; lane 3 = 14  $\mu\text{g}$ , specific activity 886; lane 4 = 24  $\mu\text{g}$ , specific activity 750; lane 5 = 10  $\mu\text{g}$ , specific activity 556. The immunoblotting analysis in B, lanes 1-3, contained AMP deaminase, specific activity 78, purified from *E. coli* HB101 (EBS-6). Lane 1 = 22  $\mu\text{g}$ , lane 2 = 4.5  $\mu\text{g}$ , and lane 3 = 2.2  $\mu\text{g}$ . Lane 4 contained 0.9  $\mu\text{g}$  of purified proteolyzed AMP deaminase, specific activity 316. Molecular weight analysis of the proteolyzed AMP deaminase and native AMP deaminase is shown in C. Molecular mass markers at the indicated molecular masses were soybean trypsin inhibitor (20 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase B (97 kDa), and *E. coli*  $\beta$ -galactosidase (116 kDa) as described under "Methods."

than 5% of the catalytic activity can be attributed to proteolyzed AMP deaminase based on immunoanalysis. When proteolysis is prevented, the resulting AMP deaminase has a subunit molecular weight of 100,000 daltons (Fig. 2C), in agreement with the molecular weight reported from the sequencing of the yeast AMD gene (19). These data establish that the lower molecular weight enzyme obtained above (Fig. 2B, lane 4;  $M_r$  86,000) and by others (28) is a proteolytic fragment of the full protein.

The partially purified large form of AMP deaminase lost 79% of its activity in a single freeze-thaw cycle. In contrast,

the proteolyzed AMP deaminase can be repeatedly frozen and thawed with only a gradual decrease in specific activity.

**Kinetic Properties of AMP Deaminase**—In the absence of ATP, the AMP saturation curve for proteolyzed AMP deaminase is sigmoidal with an  $S_{0.5}$  of  $1.35 \pm 0.03$  mM and a Hill coefficient of 2.1. Addition of ATP decreases both the  $S_{0.5}$  and the Hill coefficient (Fig. 3A). Similar kinetic parameters were obtained with enzyme preparations with the same molecular weight and with specific activities from 100 to 800  $\mu\text{mol/min/mg}$  (5, 28, 29). High ionic strength of 0.70 M KCl increased the  $S_{0.5}$  to  $8.8 \pm 0.3$  mM and decreased the Hill coefficient to 1.7. High ionic strength prevents ATP activation, but does not alter  $V_M$  values.

The kinetic properties of the large molecular weight AMP deaminase are similar to those of the proteolyzed enzyme as indicated in Fig. 3, A and B. Table IIA summarizes the kinetic parameters obtained with proteolyzed AMP deaminase and compares them with values obtained for the large molecular weight enzyme. The kinetic parameters obtained with both forms of AMP deaminase are similar. For the large form of the enzyme, the AMP saturation curve is sigmoidal with an  $S_{0.5}$  of  $2.7 \pm 0.1$  mM and a Hill coefficient of 2.1 when ATP is absent. In the presence of 0.1 mM ATP, the AMP saturation curve is hyperbolic with a  $K_M$  of  $0.50 \pm 0.04$  mM (Fig. 3B). The addition of ATP has no effect on the  $V_M$ . The kinetic parameters obtained with the large molecular weight AMP deaminase are summarized in Table IIB. The proteolyzed form of AMP deaminase was used in subsequent experiments to characterize the kinetic mechanism.

**Activation by ATP, MgATP, and ADP**—The effect of ATP is to decrease the  $S_{0.5}$  for AMP to 0.3 mM and the Hill coefficient to 1.0 as ATP approaches saturation. A replot of the  $S_{0.5}$  values for AMP as a function of ATP concentration gives an apparent dissociation constant of 6  $\mu\text{M}$  for ATP (Fig. 4). Activation of yeast AMP deaminase also occurs with ADP in a manner similar to that observed for ATP. The  $S_{0.5}$  for AMP decreases to 0.5 mM and the Hill coefficient decreases to 1.0 as ADP approaches saturation. A replot of the ADP activation data gave an apparent dissociation constant of 150  $\mu\text{M}$  for ADP (data not shown).

Activation with both ADP and ATP occurred without added metal ions, suggesting that free nucleotides activate (see also Refs. 5 and 28). Since most nucleotide-requiring proteins bind a metal-ATP complex, activation by free ATP was verified

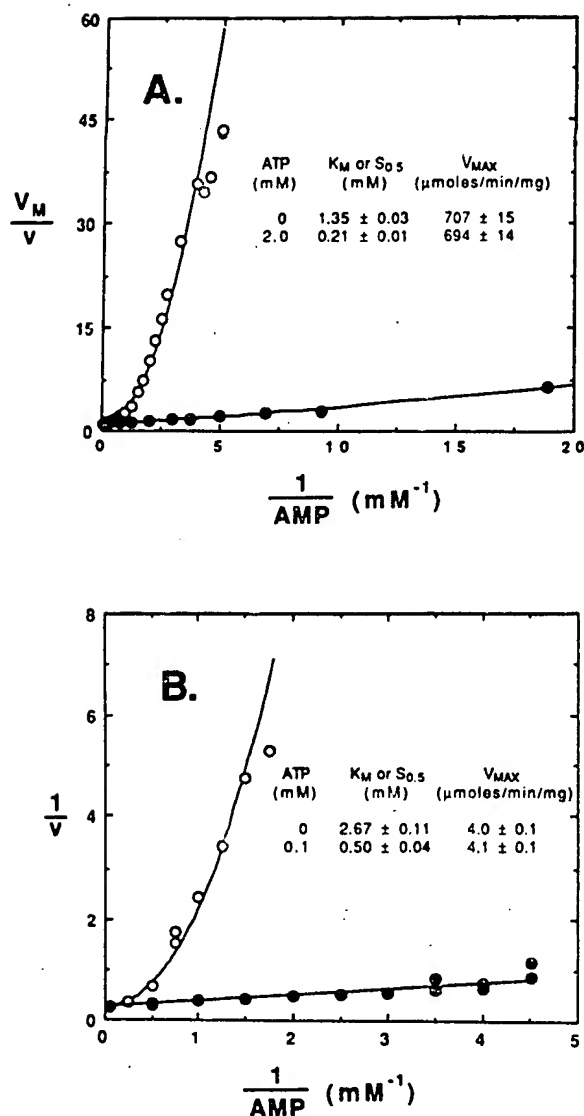


FIG. 3. Deamination of AMP by the proteolyzed (A) and native (B) forms of yeast AMP deaminase. The reactions were at 30°C in 50 mM triethanolamine HCl, pH 7.0, 100 mM KCl, 100  $\mu$ M DTT. Initial rates were obtained with both forms in the presence (●) and absence of ATP (○). The lines are the best least squares fits to the data as outlined under "Methods." The enzyme used for B is described in Table IB. The specific activity of the preparation dropped to 4 after a single freeze-thaw cycle.

by studies in the presence of EDTA. The reaction rate at 0.4 mM AMP and 0.2 mM ATP was independent of EDTA concentration to 12 mM EDTA (data not shown). Free Mg(II) inhibited the enzyme while the MgATP complex activates with AMP fixed at 2.0 mM (near the  $S_{0.5}$  value) (Fig. 5). Under these conditions, the apparent dissociation constant for free ATP was 8  $\mu$ M (similar to the data presented in Fig. 4), whereas that for MgATP was 80  $\mu$ M.

The ability of MgATP to activate AMP deaminase was evaluated by fixing total MgCl<sub>2</sub> at 1.0 mM and varying ATP. Using a dissociation constant of 7  $\mu$ M for the MgATP complex (see legend, Fig. 5) free ATP is 0.8  $\mu$ M when MgATP is present at 100  $\mu$ M. Note that 100  $\mu$ M MgATP caused a 2.5-fold increase in rate, whereas 0.8  $\mu$ M ATP causes only a slight (less than 10%) increase in rate. Additional experiments, with MgCl<sub>2</sub> fixed at 5 mM, which further prevents free ATP for-

TABLE II

Kinetic constants for proteolyzed and native AMP deaminase

Kinetic experiments were performed at 30°C by adding a small aliquot of enzyme to 50 mM triethanolamine HCl or 10 mM HEPES, pH 7.0, 100 mM KCl, 100  $\mu$ M DTT containing the appropriate concentrations of AMP and ATP. Kinetic constants are reported as the weighted mean  $\pm$  standard error (46).

ATP <sup>a</sup>	$K_M$ or $S_{0.5}$	$V_M^b$	$n^c$	$\mu^d$
A. Proteolyzed AMP deaminase				
mM	mM	$\mu$ mol/min/mg		M
0	$1.35 \pm 0.03$	$707 \pm 15$	2.1	0.15
0.1	$0.30 \pm 0.01$	$627 \pm 155$	1.0	0.15
2.0	$0.21 \pm 0.01$	$694 \pm 14$	1.3	0.15
0	$8.8 \pm 0.3$	$1076 \pm 28$	1.7	0.70 <sup>e</sup>
0.1	$13.5 \pm 0.4$	$1298 \pm 30$	1.2	0.70
B. Large AMP deaminase				
0	$2.67 \pm 0.11$	$4.0 \pm 0.1$	2.1	0.15
0.1	$0.50 \pm 0.04$	$4.1 \pm 0.1$	1.0	0.15

<sup>a</sup> ATP concentration in the assay mix.

<sup>b</sup>  $V_M$  values varied with age and purity of the enzyme sample. The values for 0 and 2.0 mM ATP were obtained with enzyme of the same specific activity. The value for 0.1 mM ATP is the mean for six determinations  $\pm$  the standard deviation from four different enzyme preparations.

<sup>c</sup> Hill coefficient.

<sup>d</sup> In initial experiments, ionic strength varied from 0.15 to 0.2 M. This variation had no significant effect on the kinetic parameters.

<sup>e</sup> KCl was added at 0.65 M to increase  $\mu$  to 0.70 M.

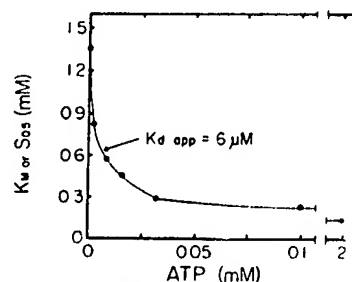


FIG. 4.  $S_{0.5}$  values of AMP deamination as a function of ATP concentration. Initial rates were measured at 30°C in 50 mM triethanolamine HCl, pH 7.0, 100 mM KCl, 100  $\mu$ M DTT, and the indicated concentration of ATP. The line was drawn by eye to fit the experimental points.

mation, gave similar results. These experiments establish that MgATP is capable of activating yeast AMP deaminase. The apparent inhibition constant for free Mg(II) was 0.3 mM in the absence of added ATP.

**Allosteric and Product Inhibition Studies**—Both GTP and PO<sub>4</sub> are inhibitors of AMP deaminase from yeast (5, 18, 28) and other sources (30–33). The inhibition patterns for both GTP and PO<sub>4</sub> were competitive with respect to AMP in the presence and absence of ATP (see Fig. 6 for GTP inhibition, PO<sub>4</sub> data was similar and is not shown). With 0.1 mM ATP, the data gave a  $K_i$  of  $48 \pm 3$   $\mu$ M for GTP and  $0.35 \pm 0.12$  mM for PO<sub>4</sub>. In the absence of ATP, the inhibition constants increased to approximately 0.4 mM for GTP and 3.0 mM for PO<sub>4</sub>. The decrease in the inhibition constants for GTP and PO<sub>4</sub> in the presence of ATP indicates that GTP and PO<sub>4</sub> bind at sites separate from ATP.

The products IMP and NH<sub>3</sub> inhibit AMP deaminase competitively (Fig. 7). A  $K_i$  of  $\sim 4.7$  mM was measured for IMP and a  $K_i$  of  $\sim 3$  mM for ammonia.<sup>3</sup> Competitive inhibition by

<sup>3</sup> The concentration of NH<sub>4</sub>Cl required to double the  $S_{0.5}$  for AMP deamination is  $\sim 0.5$  M. This corresponds to  $\sim 3$  mM NH<sub>3</sub> at pH 7.0 using a  $pK_a$  of 9.2 for NH<sub>3</sub>.

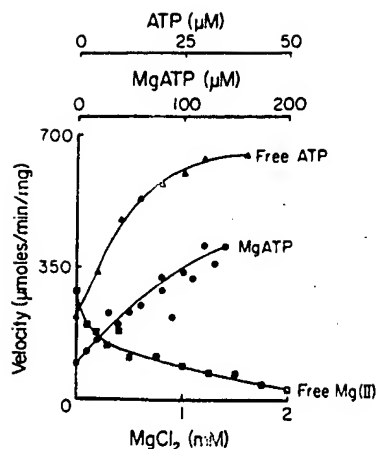


FIG. 5. The effect of free Mg(II), MgATP, and ATP on the catalytic activity of yeast AMP deaminase. The AMP concentration was fixed at 2 mM. The total MgCl<sub>2</sub> concentration was fixed at 0 and 1 mM, respectively, for the curves labeled "free ATP" and "MgATP." No ATP was added for the curve labeled "free Mg(II)." The concentration of MgATP was calculated using the known dissociation constant of Mg(II) to ATP (47). The lines were drawn by eye to fit the experimental points.

both products indicates that yeast AMP deaminase is in rapid equilibrium with AMP before catalysis occurs and that product release is random. The results of the inhibition studies are summarized in Table III.

For a rapid equilibrium random mechanism with both products present, the initial velocity expression is:

$$v = \frac{V_M[AMP]}{K_M \left( 1 + \frac{[NH_3]}{K_{NH_3}} + \frac{[IMP]}{K_{IMP}} + \frac{[NH_3][IMP]}{K_{NH_3}K_{NH_3-IMP}} \right) + [S]} \quad (4)$$

where  $K_{\text{subscript}}$  represents the dissociation constant of the subscripted ligand from the subscripted ligand-enzyme complex.  $K_{NH_3-IMP}$  represents the dissociation constant for IMP from the  $E \cdot NH_3$  complex. When  $[AMP]$  and  $NH_3$  are fixed, Equation 4 can be rearranged to:

$$\frac{1}{v} = \frac{B}{A} + \frac{[IMP]}{A} + \left( \frac{1}{K_{IMP}} + \frac{[NH_3]}{K_{NH_3}K_{NH_3-IMP}} \right) \quad (5)$$

where  $A = \frac{V_M[AMP]}{K_M}$  and  $B = 1 + \frac{[AMP]}{K_M} + \frac{[NH_3]}{K_{NH_3}}$ . A plot

of  $\frac{1}{v}$  versus  $[IMP]$  at constant  $[AMP]$  and  $[NH_3]$  provides a value for  $K_{NH_3-IMP}$ , since all the other constants are known. Such an experiment is shown in Fig. 8 resulting in a  $K_{NH_3-IMP}$  of 25 mM. Using this value and the values for  $K_{IMP}$  and  $K_{NH_3}$ , a value of 16 mM can be calculated for  $K_{IMP-NH_3}$ , the dissociation constant for  $NH_3$  from the  $E \cdot IMP$  complex.

**Attempts to Demonstrate the Reverse Reaction**—The reverse reaction of AMP deaminase was attempted with the use of AMP nucleotidase ( $AMP + H_2O \rightarrow \text{adenine} + \text{ribose 5-phosphate}$ ) as a coupling enzyme to remove AMP formed from IMP and  $NH_3$ . AMP which is formed would be converted to adenine and analyzed by HPLC as outlined under "Methods." A variety of conditions used in attempts to demonstrate the reverse reaction are summarized in Table IV. No reverse reaction was detected under any of these conditions. In the experiments utilizing myokinase and pyruvate kinase to remove AMP, reaction mixtures contained 0.1  $\mu\text{Ci}$  of  $[8-^{14}\text{C}]$  IMP and the ATP pool monitored for the appearance of radioactivity. ATP was separated from IMP as described in

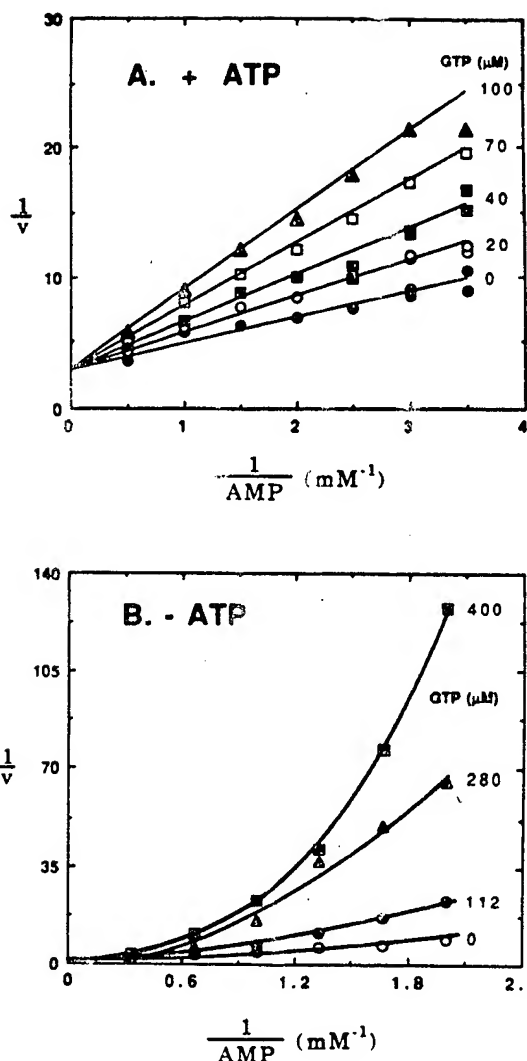


FIG. 6. Inhibition of AMP deamination by GTP in the presence (A) or absence (B) of 100  $\mu\text{M}$  ATP. Initial rates were measured at 30°C in 50 mM triethanolamine HCl, pH 7.0, 100 mM KCl, and 100  $\mu\text{M}$  DTT. The points are the experimental initial rates and the lines are drawn with the use of constants obtained by computer fit to Equation 1 (A) or by eye (B).

Ref. 34. Based on the results summarized in Table IV, the reverse rate is  $< 1 \times 10^{-4}$   $\mu\text{mol/min/mg}$ .

#### DISCUSSION

The purpose of this study was to 1) establish the role of AMP deaminase in AMP catabolism in cell-free yeast extract, 2) develop a large-scale purification, 3) characterize the kinetic mechanism and regulatory properties of the enzyme, and 4) establish the effect of the loss of the N-terminal 192 amino acids on the kinetic properties of the enzyme. AMP deaminase is the first and major step in the pathway for the conversion of AMP to IMP in cell free extracts of bakers' yeast. This role has also been proposed for other biological systems (11, 35). AMP deaminase is not essential for yeast growth under normal conditions, although cells deficient in the enzyme have an increased generation time (19). In mammalian systems the enzyme is involved in the maintenance of the adenylate energy charge (7, 8) and the operation of the purine nucleotide cycle (10). The muscle enzyme is also not

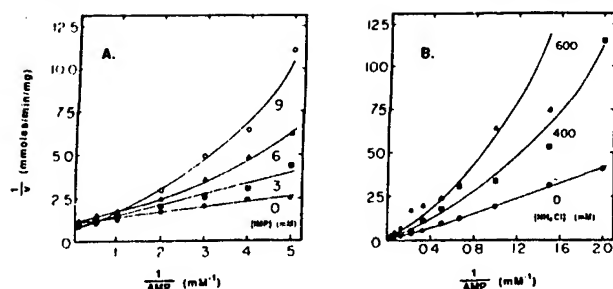


FIG. 7. Product inhibition of yeast AMP deaminase. A, inhibition by IMP. Reactions were carried out at 30°C in 10 mM HEPES, pH 7.0, 100 mM KCl, 100  $\mu$ M ATP, 100  $\mu$ M DTT, and the indicated millimolar concentrations of IMP. B, Inhibition by  $\text{NH}_4\text{Cl}$ . Reactions were carried out at 30°C in 50 mM triethanolamine HCl, pH 7.0, 100  $\mu$ M ATP, 100  $\mu$ M DTT, and the indicated millimolar concentrations of  $\text{NH}_4\text{Cl}$ . Ionic strength was maintained at 0.70 M by varying the KCl concentration. The lines drawn in both figures were drawn by eye to fit the experimental points.

TABLE III  
Competitive inhibitors versus AMP

Inhibitor	ATP <sup>a</sup> mM	$K_i^b$ $\mu$ M
GTP	0	400
GTP	0.1	48 $\pm$ 3
$\text{PO}_4$	0	3000
$\text{PO}_4$	0.1	350 $\pm$ 120
IMP	0.1	4700
$\text{NH}_4$	0.1	3000

<sup>a</sup> ATP concentration in the assay mix.

<sup>b</sup> In the presence of ATP, the reciprocal plots for AMP deamination are linear and the inhibition constants are from fits to Equation 3. In the absence of ATP, the reciprocal plots are nonlinear, and the inhibition constants represent the concentration of inhibitor necessary to double the  $S_{0.5}$  for AMP.

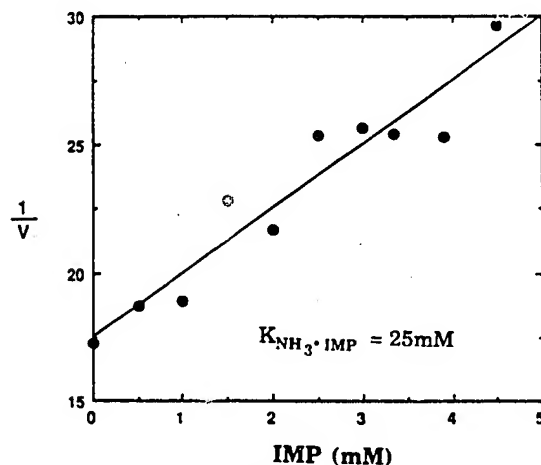


FIG. 8. Determination of  $K_{\text{NH}_3\text{-IMP}}$ . Initial rates as a function of IMP were determined at 30°C in 50 mM triethanolamine HCl, pH 7.0, 100 mM KCl, 100  $\mu$ M DTT, 100  $\mu$ M ATP, 500  $\mu$ M AMP, 200 mM  $\text{NH}_4\text{Cl}$ . The line is a linear regression fit to the experimental points.

essential for survival, since humans deficient in AMP deaminase experience relatively minor symptoms and muscle biopsies from such people appear virtually normal (36). Trypanosomes, which do not carry out *de novo* synthesis of purines, appear to lack both adenine and adenosine deaminases (37, 38), suggesting that AMP deaminase may play an important metabolic role in these organisms.

**Purification and Proteolysis of AMP Deaminase**—The sequential elution of AMP deaminase from phosphocellulose by KCl and ATP provides an efficient method to purify the enzyme. Enzyme isolated in this manner has a subunit molecular weight of  $M_r$  86,000 and has been proteolytically cleaved from the larger, native form. Rapidly growing yeast (19) or expression of yeast AMP deaminase in *E. coli* HB101 (EBS-6) produces a yeast AMP deaminase with a subunit molecular weight of 100,000. However, the enzyme is unstable and is readily proteolyzed to the  $M_r$  86,000 form. Despite the continuous presence of inhibitors, protein purified to homogeneity always has a subunit molecular weight of 86,000. Partially purified large enzyme lost 80% of its activity in a single freeze-thaw cycle and was unstable at 4°C, conditions which do not inactivate the proteolytically cleaved enzyme. Proteolytic sensitivity appears to be a general property of AMP deaminases, since proteolysis also occurs during the purification of rat muscle AMP deaminase (39). However, the results with AMP deaminase from yeast clearly establish that the functional, *in vivo* form of the enzyme is the large molecular weight form.<sup>4</sup>

**The Regulatory Mechanism of AMP Deaminase**—Purified AMP deaminase exhibits a sigmoidal AMP saturation curve with a  $S_{0.5}$  of  $1.35 \pm 0.03$  mM and a Hill coefficient of 2.1. Both ATP and ADP, in the absence of metal ions, decrease the Hill coefficient to 1.0 and activate the enzyme by decreasing the  $K_M$  to  $0.21 \pm 0.01$  mM without affecting the maximum catalytic rate. The kinetic properties of the large form of AMP deaminase are similar, except that the  $S_{0.5}$  for AMP is approximately 2-fold higher both in the presence and absence of ATP. Since both forms of AMP deaminase have similar kinetic constants for substrate and allosteric activation, the  $M_r$  14,000 N terminus of the enzyme cannot be responsible for catalytic activity or allosteric regulation.

Yoshino and Murakami (40) have reported that free  $\text{Mg}(\text{II})$  ions activate purified yeast AMP deaminase. However, the results of Fig. 5 clearly establish that free  $\text{Mg}(\text{II})$  inhibits the enzyme. The activation constant for  $\text{MgATP}$  is about 10-fold higher than the value for ATP. These results suggest that both ATP and  $\text{MgATP}$  bind at the allosteric site to activate the enzyme, but that  $\text{Mg}(\text{II})$  binding alone is inhibitory. A separate binding site for free divalent metal ion cannot be eliminated by the kinetic results.

Both inorganic phosphate and GTP are competitive inhibitors of AMP, consistent with their binding at sites which abolish AMP binding. These sites are not necessarily the AMP binding sites. Activation by ATP causes an increased affinity of the enzyme for AMP and also increased its affinity for GTP and  $\text{PO}_4$ . Since the inhibition constants for GTP and  $\text{PO}_4$  decrease when ATP is near saturation, GTP and  $\text{PO}_4$  appear to bind at sites separate from ATP.

Similarities between the regulatory properties of AMP nucleosidase from *A. vinelandii* (41, 42) and yeast AMP deaminase has led to the proposal for similar metabolic functions. Both enzymes are activated by ATP and inhibited by  $\text{PO}_4$ . At saturating ATP, their  $K_M$  values (0.11 mM for AMP nucleosidase and 0.21 mM for AMP deaminase) are similar.

<sup>4</sup> Meyer *et al.* (19) have constructed a yeast strain which lacks AMP deaminase. Activity was restored in this strain by inserting a YEp plasmid construct containing the AMD gene. This strain was designated SM4-40(YEpAMD-16). In crude extracts of SM4-40(YEpAMD-16), the large form of AMP deaminase represents 75–90% of the immunodetectable enzyme (see Fig. 2B, lanes 3–5, in Ref. 19). The specific activity of AMP deaminase in SM4-40(YEpAMD-16) crude extracts is  $2.06 \pm 0.55$ , whereas the specific activity of crude extracts from bakers' yeast is  $0.12 \pm 0.01$ . The ratio of these values is 17, which corresponds to the copy number of the plasmid coding for the AMD gene.

TABLE IV  
Reverse reaction conditions

Assay mix <sup>a</sup>	Incubation time	Incubation temperature	NH <sub>4</sub> Cl	IMP	AMP deaminase <sup>b</sup>	Coupling enzyme
	h	°C	mM		units	
RR1	Overnight	25	2000	125	1.8	None
RR2	30	37	50	0.5	22	0.03 unit AMP nucleosidase <sup>c</sup>
RR3	4	25	20	1.0	40	2.2 units AMP nucleosidase <sup>c</sup>
RR4	5	30	300	20	154	0.6 unit AMP nucleosidase <sup>d</sup>
RR5	25	30	56	1.5	15	1.7 units AMP nucleosidase <sup>d</sup>
RR6	Overnight	30	1500 <sup>e</sup>	5	18	0.7 unit myokinase and 0.4 unit pyruvate kinase
RR7	Overnight	30	1200 <sup>e</sup>	16	9	14 units myokinase and 8 units pyruvate kinase

<sup>a</sup> The assay mixes are: RR1, 50 mM CHES, pH 9.0, 100  $\mu$ M DTT; RR2, 20 mM HEPES, pH 8.5, 100 mM KCl, 100  $\mu$ M DTT, 20 mM sodium tripolyphosphate, 2.4 mM MgCl<sub>2</sub>; RR3, 20 mM HEPES, pH 8.5, 100 mM KCl, 100  $\mu$ M DTT, 2 mM MgATP; RR4, 50 mM Tricine, pH 8.6, 100 mM KCl, 100  $\mu$ M DTT, 1 mM MgATP; RR5, 20 mM Tricine, pH 8.5, 20 mM KCl, 100  $\mu$ M DTT, 20 mM sodium tripolyphosphate, 2.4 mM MgCl<sub>2</sub>; RR6, 50 mM glycine, pH 9.5, 5 mM KCl, 100  $\mu$ M DTT, 1 mM ATP, 10 mM phosphoenolpyruvate; RR7, 54 mM glycine, pH 9.5, 9 mM KCl, 100  $\mu$ M DTT, 2.1 mM ATP, 2.1 mM phosphoenolpyruvate.

<sup>b</sup> Amount of yeast AMP deaminase added to the reverse reaction assay mix. The specific activities of the AMP deaminase preparations used varied from 400 to 600  $\mu$ mol/min/mg.

<sup>c</sup> *A. vinelandii* AMP nucleosidase was the coupling enzyme. MgATP and sodium tripolyphosphate are activators of AMP nucleosidase (41).

<sup>d</sup> *E. coli* AMP nucleosidase was the coupling enzyme.

<sup>e</sup> 0.1  $\mu$ Ci of [8-<sup>14</sup>C]IMP was included as a tracer.

sidase and 0.21 mM for AMP deaminase) are similar. These data and the roles of the enzymes in degrading AMP in cell extracts indicate that the enzymes have similar metabolic roles, although they show essentially no amino acid sequence similarity (19).

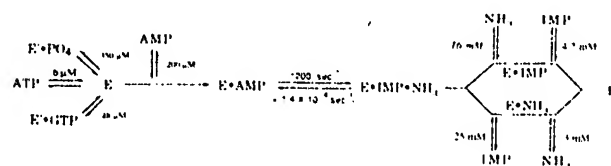
**Kinetic Mechanism of AMP Deaminase**—A summary of the steady-state mechanism and the kinetic and dissociation constants associated with the AMP deaminase-ATP complex is provided in Scheme II. The competitive product inhibition patterns for both IMP and NH<sub>3</sub> are consistent with a rapid equilibrium mechanism. This finding is consistent with the expression of primary <sup>15</sup>N V/K kinetic isotope effects by yeast AMP deaminase (43). Ashby and Frieden (44) have also postulated a rapid equilibrium mechanism for rabbit muscle AMP deaminase. In a rapid equilibrium mechanism, the observed kinetic constants are dissociation constants. Competitive inhibition between AMP and PO<sub>4</sub>, as well as AMP and GTP indicate that PO<sub>4</sub> and GTP either 1) compete for the AMP site; 2) bind at sites distinct from the AMP site, but in a mutually exclusive manner; or 3) bind a distinct site which interferes with AMP catalysis but not binding. The structural diversity of these three compounds suggests that option 1 is unlikely; however, kinetic results cannot distinguish among the various possibilities. The observation that ATP increases the affinity of AMP 6.4-fold, GTP by 8.3-fold, and PO<sub>4</sub> by 8.6-fold suggests a common mechanism for these changes.

The forward rate constant for yeast AMP deaminase is 1200 s<sup>-1</sup> and  $V_M/K_M = 6 \times 10^6$  M<sup>-1</sup> s<sup>-1</sup>. Although this is

approaching within several orders of magnitude the diffusion controlled limit of  $\sim 10^9$  M<sup>-1</sup> s<sup>-1</sup> for substrates the size of AMP, the catalytic step must be relatively slow compared to the binding and release of AMP. An upper limit for the internal equilibrium constant can be estimated from the forward reaction rate and the limit on the reverse rate. For  $K_{\text{internal}} = \frac{[E \cdot \text{IMP} \cdot \text{NH}_3]}{[E \cdot \text{AMP} \cdot \text{H}_2\text{O}]}$ , the ratio is  $> 8.6 \times 10^6$  and the  $\Delta G_{\text{internal}} < -6.9$  kcal/mol. Although the yeast AMP deaminase reaction could not be reversed under a variety of conditions (Table IV), Wolfenden (45) reported the formation of 10–60 cpm of [8-<sup>14</sup>C]adenosine from 8800 to 12500 cpm of [8-<sup>14</sup>C]inosine using mammalian or fungal adenosine deaminase at pH values of 9.1–9.3 and ammonia concentrations of 0.5–2.0 M. From these results, Wolfenden (45) calculated that the apparent free energy of adenosine hydrolysis at pH 7 is -5.4 kcal/mol and estimated the free energy of AMP hydrolysis to be -6 kcal/mol at pH 7 using reported equilibrium measurements from several reactions. These free energy measurements are similar to the estimate  $\Delta G_{\text{internal}}$  for yeast AMP deaminase; however, the reaction of yeast AMP deaminase is not reversible under a variety of conditions.

Competitive inhibition between AMP and both products, IMP and NH<sub>3</sub>, indicate that product release is random. By measuring inhibition by IMP at fixed concentrations of NH<sub>3</sub> and AMP, the dissociation constant for IMP from the *E*·NH<sub>3</sub> complex was measured and the dissociation constant for NH<sub>3</sub> from the *E*·IMP complex calculated. These data show that IMP and NH<sub>3</sub> bind to enzyme tighter in binary complexes than they bind to the binary complexes to form the ternary *E*·IMP·NH<sub>3</sub> complex.

**Conclusions**—Despite the extensive literature on the regulation of adenylate metabolism, the process is poorly understood. Yeast provides a good example of these problems, since genetic interruption of AMP deaminase results in cells which grow, but at reduced rates. Characterization of both the genetic (19) and catalytic regulatory mechanisms of yeast AMP deaminase defines a simple eukaryotic system for understanding adenylate regulation. Regulation of AMP deaminase activity by metabolite controls is implied by the demonstration that yeast contains a single, constitutive gene for the enzyme



SCHEME II. Kinetic mechanism for reaction catalyzed by AMP deaminase in the presence of ATP. The numbers represent dissociation constants for the release of individual reactants or the microscopic rate constants. The italicized constant is calculated from the other experimentally determined constants. *E* represents AMP deaminase saturated with ATP, and *E'* represents the enzyme without ATP.



(19). The metabolic and kinetic studies presented here provide the following advances in understanding the regulation of adenylate metabolism in yeast: 1) AMP deaminase is the only enzyme which degrades AMP in cell-free extracts of yeast. 2) Purified enzyme which has lost the N-terminal 192 amino acids by proteolysis has unchanged kinetic properties for substrate and allosteric activator. 3) A procedure has been developed for the isolation of 100-mg quantities of AMP deaminase. 4) AMP deaminase follows a rapid equilibrium random reaction pathway where the hydrolytic step is experimentally irreversible. The internal (on the enzyme) equilibrium lies far in the direction of products. 5) Allosteric activation occurs with free ATP and less well by ADP and MgATP. GTP and PO<sub>4</sub> are allosteric inhibitors.

These results provide experimental information required for further metabolic studies in yeast or mechanistic studies to establish the catalytic mechanism of AMP deaminase.

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## IDENTIFICATION OF FUNCTIONAL DOMAINS IN AMPD1 BY MUTATIONAL ANALYSIS

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Received October 28, 1994

**SUMMARY:** AMP deaminase (AMPD) is a complex allosteric enzyme encoded by a multigene family in higher eukaryotes. The amino terminus of each AMPD gene is unique, while the mid and carboxy termini have been highly conserved among all the AMPD genes. Mutational analyses of the AMPD1 gene demonstrate that the catalytic site and a regulatory site, likely an ATP binding site, are located in the highly conserved carboxy terminus. Deletion mutants and a normal splice variant of AMPD1 demonstrate that the amino terminus has a profound influence on catalytic activity of AMPD and by inference from prior studies this region also influences binding of AMPD1 to myosin. Results of these studies suggest a regulatory model in which alternative splicing in the amino terminal region of AMPD1 generates isoforms of AMPD that exhibit differential sensitivity to effector molecules such as ATP. © 1994 Academic Press, Inc.

AMP deaminase (AMP aminohydrolase; E.C. 3.5.4.6.; AMPD) is a ubiquitous enzyme found in all eukaryotic cells, and it has been extensively studied because of its complex allosteric regulatory properties (1-5), binding to intracellular organelles (6-8), and association with clinical syndromes related to inherited defects of this enzyme activity (9-11). This enzyme activity is encoded by a multigene family in higher eukaryotes (12), and some clues to potential structure/function relationships in this enzyme have been suggested. The comparisons of primary structure suggest the unique amino terminus of the different AMPD gene products may encode isoform specific characteristics for the various AMPD peptides, and the conserved mid and carboxy terminal regions may harbor the catalytic as well as other potential regulatory sites (12-14).

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### Abbreviations:

AMP: adenosine monophosphate, AMPD: AMP deaminase, dNTP: deoxynucleotide triphosphate, PCR: polymerase chain reaction, UTR: untranslated region.

To test these assumptions on structure/function relationships, deletions and point mutations have been introduced into the respective regions of AMPD1 cDNAs. Rat AMPD1 cDNA was used for the deletion analyses of the amino terminus because of the availability of cDNA clones which contain and exclude exon 2, the site of alternative splicing of the AMPD1 gene product (15). Human AMPD1 cDNA was used for the point mutation analyses of the mid and carboxy terminal regions because of the potential applicability of these results to inherited and acquired deficiency of AMPD activity in skeletal muscle, a common finding in biopsies of human muscle (9). The high degree of conservation between rat and human AMPD1 suggests the conclusions obtained for one species will also apply to the other.

## EXPERIMENTAL PROCEDURES

### AMPD activity assay

AMPD activity was assayed radiochemically ( $^{14}\text{C}$ -AMP from Amersham, United Kingdom)(16) and through the quantitation of IMP by HPLC (Waters Millipore Corp. U.S.A.), as described previously (17).

### Construction of rat AMPD expression vector

The expression vector pKK233-2 (pKK; Pharmacia Biosystems, Piscataway, NJ) (18) was used for prokaryotic expression of AMPD1 peptides. A pBS-derived plasmid, pBSrAMPD1, containing the full length AMPD cDNA, was isolated as described previously (19). Various deletion constructs of this AMPD expression vector (pKKrAMPD, pKKrAMPD $\Delta$ exon2, pKKrAMPD $\Delta$ 67, pKKrAMPD $\Delta$ 177, pKKrAMPD $\Delta$ 305) were made using standard restriction enzyme digestion techniques, and the resulting constructs, all confirmed by sequencing, are shown in Figure 1.

### Random mutagenesis of human AMPD1 cDNA using PCR

Human cDNA of AMPD1 was obtained by PCR as described previously (10). PCR was carried out in a standard buffer (50 mM KCl, 10 mM Tris/Cl, pH 8.4, 0.1 mg/ml gelatin), with 20 U/ml Taq polymerase under two different conditions: 1.8 mM  $\text{MgCl}_2$ , 200 nM of each dNTP (condition A) and 4 mM  $\text{MgCl}_2$ , 1 mM of each dNTP (condition B). Preliminary experiments demonstrated that condition A and condition B generated on average one base change per 810 and 1380 nucleotides, respectively. The 5' and 3' regions of human AMPD1 were then ligated in the correct orientation in the pKK233-2 prokaryotic expression vector described above.

### Prokaryotic expression and purification of AMPD1 protein and Western blots

*E. coli* JM105 as transformed with pKK expression vectors containing various AMPD1 constructs (20). IPTG was added to a final concentration of 1.5 mM. Bacteria were harvested, the pellet washed in cold phosphate buffered saline, resuspended and sonicated. AMPD activity was assayed in crude lysate and in lysate dialyzed overnight against 50 mM imidazole/HCl, pH 6.5, 150 mM KCl, 1 mM dithiothreitol. AMPD activity in lysates of *E. coli* transformed with the pKK vector alone was < 0.1 mIU/mg (21).

AMPD protein was purified from bacterial cell lysate using phosphocellulose column chromatography (P11 cellulose phosphate from Whatman Chemical Separation Ltd, England) with modification of the method described by Smiley et al. (22).

Western blots were performed as described before (16).

### In vitro transcription and translation of AMPD1

Several RNAs were synthesized from cDNAs corresponding to the full length cDNA or 5' deleted cDNAs of AMPD (Figure 1) using the T7 promoter in pBS. In vitro transcribed RNA (0.3 g) was translated in a 25  $\mu\text{l}$  of reaction mixture (17.5  $\mu\text{l}$  nuclease treated rabbit reticulocyte lysate, 5  $\mu\text{l}$  RNasin, 0.5  $\mu\text{l}$  1 mM amino acid mixture without methionine, 2  $\mu\text{l}$   $^{35}\text{S}$  methionine (20  $\mu\text{Ci}$  4.5  $\mu\text{l}$  mRNA) at 30  $^\circ\text{C}$  for 1 hr.

Analysis of in vitro proteolysis was performed by incubating 10  $\mu\text{l}$  of in vitro translated AMPD peptides with rat skeletal muscle lysate at 37  $^\circ\text{C}$  overnight. SDS-PAGE analysis (23) was performed after incubation.

### Computer analysis

Computer analyses were performed using the Microvax computer of the Cancer Center of Duke University Medical Center, Durham, NC. Sequences of the NBRF Nucleic Acid and Protein Sequence Data Bank were analyzed with the Sequence Analysis Software Package of the Genetics Computer Group Version 6.0 (24).



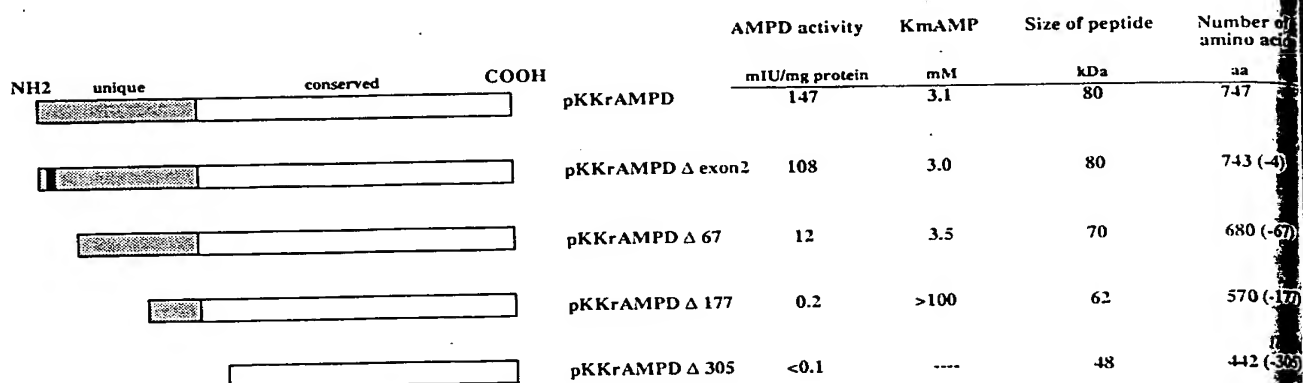


Figure 1. Prokaryotic expression of rat AMPD cDNAs. cDNAs for rat AMPD1 (pKKrAMPD, pKKrAMPD $\Delta$ exon2, pKKrAMPD $\Delta$ 67, pKKrAMPD $\Delta$ 177, pKKrAMPD $\Delta$ 305) are illustrated on the left. Shaded areas show unique and open areas show conserved regions. These cDNAs were ligated into a prokaryotic expression vector for transformation into *E. coli* JM105. AMPD activity, apparent Km for AMP, and peptide size estimated by SDS-PAGE gel electrophoresis and Western blot are shown on the right. Total number of amino acids in the various peptide and number of deleted amino acids are also shown in this figure.

## RESULTS

### Deletions in the unique amino terminal region of rat AMPD1

Figure 1 illustrates the number of residues removed from the AMPD1 peptide in the various deletion constructs (pKKrAMPD $\Delta$ 67, pKKrAMPD $\Delta$ 177, pKKrAMPD $\Delta$ 305), the resulting size of the AMPD peptide, and the AMPD enzyme activity in the extracts from cells transformed with the respective constructs. The AMPD peptide which excludes exon 2 encoded sequences is approximately 25 % less active than the peptide which includes these sequences, and there is no apparent difference in substrate affinity when assayed in bacterial lysates. Deletion of the amino terminal 67 amino acids (pKKrAMPD $\Delta$ 67) reduces catalytic activity of AMPD in extracts by approximately 90 % with minimal change in substrate affinity. When 177 residues were deleted from the amino terminus, or when 74% of the unique region (240 residues) was eliminated, AMPD activity is reduced by greater than 99 % but enzyme is still detectable. This truncated peptide exhibits a decrease in substrate affinity for AMP. A deletion which extends 65 residues into the conserved region of AMPD by removal of the amino terminal 305 residues yields an AMPD1 peptide with no detectable catalytic activity, even when assayed at 100 mM AMP.

Prior studies with yeast AMPD have shown that the amino terminus of this peptide is not essential for catalytic activity (25). Rat skeletal muscle AMPD is also subject to proteolysis without complete loss of catalytic activity (26), but it has not been established whether the amino terminus of AMPD1 is the site of *in vitro* proteolysis. We have utilized the deletion constructs described above to address the question of what region of rat AMPD1 is subject to proteolysis in muscle extract since this truncation influences binding of AMPD1 to myosin (26).

The deletion constructs illustrated in Figure 1 were transferred to an *in vitro* transcription plasmid to generate the corresponding mRNAs. Several AMPD1 peptides (rAMPD, rAMPD $\Delta$ 67, rAMPD $\Delta$ 177, rAMPD $\Delta$ 305) were then synthesized by *in vitro* translation. These *in vitro* synthesized,  $^{35}$ S-labeled AMPD1 peptides were incubated in rat skeletal muscle extract or

1PDΔexon2, pKKrAMPDΔ67, etc. Shaded areas show unique and translated into a prokaryotic expression system. Apparent Km for AMP, and peptide hydrolysis are shown on the right. Total released amino acids are also shown in

in the AMPD1 peptide in the various KrAMPDΔ305), the resulting size of extracts from cells transformed with the exons 2 encoded sequences includes these sequences, and there is no AMPD activity in the control bacterial lysates. Deletion of the amino terminal 305 residues of AMPD1 abrogates the AMPD activity in extracts from the transformed cells. When 177 residues were deleted from the amino terminal region (240 residues) was eliminated, the AMPD activity is still detectable. This truncated AMPD1 protein is still detectable. A deletion which extends 65 residues from the amino terminal 305 residues yields a truncated AMPD1 protein which when assayed at 100 mM AMP.

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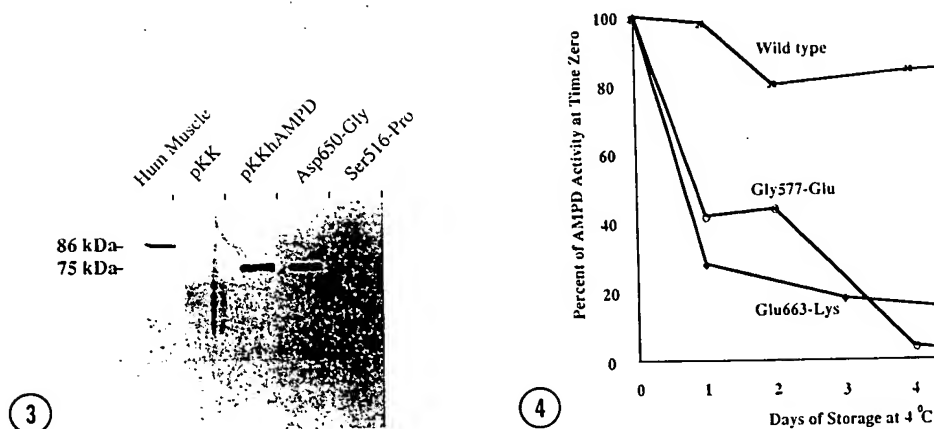
in rat skeletal muscle extract or

prior to display on SDS-PAGE, followed by autoradiography (Figure 2). The 80 kDa native rat AMPD1 <sup>35</sup>S-labeled AMPD peptide ( rAMPD ) is degraded to a 66 kDa peptide, similar to results obtained previously with AMPD isolated from rat skeletal muscle (26). In vitro synthesized AMPD peptide from which the amino terminal 67 residues are deleted ( rAMPDΔ67 ), a 70 kDa peptide, is also degraded to a 66 kDa product. The other AMPD peptides truncated to greater extents at the amino terminus ( rAMPDΔ177, rAMPDΔ305 ) are not detectably degraded following incubation in muscle extract. These results demonstrate that the target sequences in rat AMPD1 for the proteases present in rat skeletal muscle reside in the amino terminus of this peptide.

Seven *E. coli* transformants harboring a single point mutation in the mid or carboxy terminal region of human AMPD1 were identified from sequencing individual isolates derived from the PCR-based random mutagenesis ( Table 1 ). Three of these point mutations ( Phe<sup>266</sup>-Leu,

mutated nucleotide	amino acid substitution	activity in crude lysate	activity after dialysis	detection of protein in Western blot	mutation in conserved region <sup>1)</sup>
Mutations that do not alter AMPD activity					
T796-C	Phe266-Leu	yes	yes	not	no
T932-C	Asn311-Ser	yes	yes		ACM <u>N</u> QKHL <u>R</u> FIK
C1552-T	His518-Tyr	yes	yes	determined	no
Mutations that result in no enzyme activity					
A1949-G	Asp650-Gly	no	no	yes	SLSTDDP
T1545-C	Ser516-Pro	no	no	no	DSVDDE <u>S</u> K
Mutations that lead to an unstable phenotype					
G1730-A	Gly577-Glu	yes	no	not	RPHCGEAG
G1987-A	Glu663-Lys	yes	no	determined	EPLME <u>E</u> Y

1) The mutated residue is underlined.



**Figure 3.** Immunoreactivity of mutant and wild-type recombinant AMPD1 peptides.

Lane 1: fresh extract (15 mg total protein) from human muscle homogenate, not purified (positive control). Lanes 2-5: partially purified AMPD from bacterial cell lysates. Protein (100 µg) eluted from a phosphocellulose column was loaded in each lane: Lane 2 (pKK): pKK expression vector without insert (negative control); Lane 3 (pKKhAMPD): pKK containing wild-type AMPD1 cDNA; Lane 4 (Asp<sup>650</sup>-Gly): pKK containing the Asp<sup>650</sup>-Gly mutation of human AMPD1; Lane 5 (Ser<sup>516</sup>-Pro): pKK containing the Ser<sup>516</sup>-Pro mutation of human AMPD1. Molecular sizes are indicated in the margins.

**Figure 4.** Enzymatic stability of recombinant AMPD peptides.

Bacterial cell lysates for wild-type and mutant (Gly<sup>577</sup>-Glu and Glu<sup>663</sup>-Lys) AMPD were kept at 4 °C and assayed for AMPD activity at several time points. The activity for each construct is expressed as a percentage of the initial activity in this lysate. Initial activity did not vary by more than 20 % in any of the fresh lysates used for these analyses.

Asn<sup>311</sup>-Ser, His<sup>518</sup>-Tyr ) have no detectable effect on the AMPD activity measured in the bacterial lysates. Only one of these three mutations, Asn<sup>311</sup>-Ser, is located in a highly conserved sub-region (ACMNQKHLRFIK) of human AMPD1, and this mutation represents a conservative amino acid substitution. The other four point mutations resulted in an alteration in AMPD activity in the bacterial lysate, either when assayed immediately or following overnight dialysis ( Table 1 ).

One of these mutations, Asp<sup>650</sup>-Gly, is located in a highly conserved sub-region, SLSTDDP, which is very similar in sequence to the SLNTDDP domain that has been shown to be a part of the active site of the closely related enzyme, adenosine deaminase (27). This mutant produces an AMPD peptide that is easily detectable on a Western blot performed with a partially purified protein preparation isolated from bacteria transformed with this construct ( Figure 3 ). However, this mutant peptide has no detectable AMPD activity in fresh lysate or after overnight dialysis when assayed at 10 mM or 250 mM AMP.

Another mutant, Ser<sup>516</sup>-Pro, is located in a highly conserved sub-region of AMPD1, DSVDDESK, and it also yields a peptide which has no detectable AMPD activity. Unlike the Asp<sup>650</sup>-Gly mutant, this mutant does not produce a peptide that is immunologically detectable in the bacterial lysate ( Figure 3 ) so that further characterization of this mutant has not been possible.

Two other unrelated mutants produce a similar phenotype in which the fresh bacterial lysate when assayed immediately has AMPD activity comparable to that encoded by the wild-type enzyme, but following overnight dialysis no AMPD activity is detectable in lysates from the mutants. AMPD activity of the wild type peptide is unchanged after dialysis. As illustrated

Fig. 4, the AMPD activity of both these mutants is less stable than the wild-type peptide when dialyzed extract is stored for several days at 4 °C.

One of the stability mutants, Glu<sup>663</sup>-Lys, is located in a highly conserved sub-region, EPLMEEY, that has been identified as an ATP or NTP binding site based on studies of yeast AMPD (28). Thus, the phenotype of this mutant might be explained by an altered affinity of this peptide for ATP, or another NTP, with a resultant loss in enzyme activity following dialysis. Attempts to stabilize this mutant by dialysis against ATP, GTP, ADP, AMP or inorganic phosphate have not been successful, however. Since the dialyzed enzyme has no detectable enzyme activity, it has not been possible to carry out kinetic studies to determine if the allosteric properties of the mutant peptide are altered as a consequence of the Glu<sup>663</sup>-Lys mutation.

The other stability mutant, Gly<sup>577</sup>-Glu, is located in an unrelated but highly conserved sub-region of AMPD1, RHPCGEAG. The phenotype of this mutant is similar to that of the Glu<sup>663</sup>-Lys mutant, but a search of the data bank does not provide a clue as to the putative ligand that might bind to the RHPCGEAG site. Attempts to stabilize this mutant by dialysis against ATP, GTP, ADP, AMP and inorganic phosphate were without effect, and consequently assessment of the kinetic properties of this mutant have not been possible.

### DISCUSSION

Comparison of primary amino acid sequences for the various members of the AMPD multigene family suggests the mid and carboxy terminal region which is common to all isoforms contains the catalytic site as well as regulatory sites which are shared among the various isoforms. Potential functions for two subsegments in the carboxy terminal region were anticipated from prior studies with other AMP deaminases and the related enzyme adenosine deaminase. The sequence SLSTDDP is highly conserved among all the AMPD isoforms and it confirms to the predicted consensus sequence, SLSTDDP, for a binding site for six amino purines (29). Mutation of Asp<sup>650</sup> to glycine in the SLSTDDP motif of AMPD1 destroys catalytic activity, consistent with the prediction that this sequence may constitute part of the active site of this enzyme.

A second highly conserved sequence in the carboxy terminus, EPLMEEY, is predicted to be an ATP binding site from prior studies with yeast AMPD (28). Results of the present study support this prediction since the mutation of Glu<sup>663</sup> to Lys in the EPLMEEY motif of AMPD1 produces an enzyme which is very labile post-dialysis and during storage *in vitro*, a phenotype consistent with altered binding of a low molecular weight, labile molecule such as ATP. Kinetic analysis of this mutant, and the other unstable mutant (Gly<sup>577</sup>-Glu in the highly conserved RHPCGEAG motif), await development of a purification scheme which overcomes the exquisite lability of these mutants.

The amino terminal deletions of rat AMPD provide information that could not have been predicted from sequence comparisons. This region of the AMPD1 peptide has a profound influence on the catalytic activity of this enzyme as well as its binding to myosin heavy chain. These conclusions are based on the following observations: 1) deletion of the amino terminal 67 residues or deletion of exon 2 encoded sequences of AMPD1 reduces V<sub>max</sub> of this enzyme with no appreciable change in the K<sub>m</sub> for AMP, 2) *in vitro* proteolysis of recombinant AMPD1 peptides

in skeletal muscle extract is restricted to the amino terminus of this peptide, and 3) *in vitro* proteolysis of endogenous AMPD1 in skeletal muscle generates an AMPD peptide which elutes from a myosin affinity column at a 50 % fold lower ATP concentration than the native, full-length peptide (26).

These observations raise the interesting possibility that the amino terminus of AMPD1 plays a role in controlling the activity of this enzyme and/or the binding to myosin heavy chain in myocytes. These two characteristics of AMPD1 may be related since prior studies have demonstrated that AMPD binding to myosin *in vivo* responds to physiological stimulation of muscle and the catalytic properties of AMPD are altered by binding to myosin (30-32). Alternative splicing, which is restricted to the amino terminus, is controlled by stage and fiber-type specific signals in myocytes (15,21), providing a physiological mechanism for altering the primary structure of the amino terminus of AMPD1 in different types of myocytes. The latter would result in AMPD1 isoforms with different catalytic possibilities. Alternative splicing could also provide a mechanism for targeting AMPD1 to different organelles, such as the myofibril and subsynaptic junction which have been shown to co-localize with AMPD1 in myocytes (33), since the amino terminus influences binding of AMPD1 to other molecules such as myosin.

#### ACKNOWLEDGMENTS

This work was supported by grants to E.W.H. (DK12314, National Institutes of Health) and M.G. (Deutsche Forschungsgemeinschaft).

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14, National Institutes of Health)

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